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(54) Title: LACTIC ACID BATERIAL STRAINS BELONGING TO A NEW LACTOBACILLUS SPECIES AND THEIR USE IN THE FOOD AND FEED INDUSTRY

(57) Abstract: The invention relates to lactic acid bacterial strains belonging to Lactobacillus danicus, a novel Lactobacillus species isolated from Danish and Estonian cheeses and Danish dairy starter cultures. The strains are useful in theproduction of pharmaceutical, feed, sweet and food products especially in the manufacturing of different kinds of cheese for accelerating and controlling cheese ripening and flavour improvement.

LACTIC ACID BACTERIAL STRAINS BELONGING TO A NEW LACTOBACILLUS SPECIES AND THEIR USE IN THE FOOD AND FEED INDUSTRY

FIELD OF THE INVENTION

The present invention relates to the field of lactic acid bacteria and more particularly to lactic acid bacterial strains belonging to *Lactobacillus danicus*, a novel *Lactobacillus* species. Furthermore, the invention relates to the use of these bacterial strains in the food, feed, sweet and pharmaceutical industry.

TECHNICAL BACKGROUND OF THE INVENTION

Lactic acid bacteria are used extensively for fermentations in the industry, both in the manufacturing of food, feed and pharmaceutical products, including manufacturing of dairy products such as cheese, yoghurt and butter. Additionally they are increasingly used as probiotics for human and animals where it can be given as pure cultures or added or incorporated in the food or feed and even added to sweets. Cultures of such bacteria are generally referred to as starter cultures and they impart specific features to various fermented products by performing a number of functions. Lactic acid bacteria are also involved in production of food products such as in the production of fermented vegetables, cereals and meat where the use of the bacteria is not pronounced, but where their presence in the starting material for that particular food product results in a spontaneous fermentation.

In the present context, the expression "lactic acid bacteria" designates a group of Gram positive, catalase negative, non-motile, microaerophilic or anaerobic bacteria which ferment sugars with the production of acids including lactic acid as the predominantly produced acid, acetic acid, formic acid and propionic acid. The industrially most useful lactic acid bacteria are found among *Lactobacillus* species, *Lactococcus* species, *Oenococcus* species, *Streptococcus* species, *Weissella* species, *Enterococcus* species, *Leuconostoc* species and *Pediococcus* species.

As mentioned above, starter cultures of lactic acid bacteria are e.g. used in the manufacturing of cheese, a process that involves the inoculation of cheese milk with starter culture followed by a cheese ripening at low temperatures. During the initial stages of the cheese manufacturing process the starter culture rapidly depletes the lactose present in milk. The starter cell number typically decreases within one month to about 1% of the initial concentration (Peterson & Marshall, 1990) due to unfavourable conditions in the cheese during ripening such as e.g. high sodium chloride concentration, low pH and low temperature.

Most cheeses such as e.g. Cheddar require a storage period of the order of 1.5 to at least 12 months at about 4-12°C prior sale. This storage period is necessary to allow the cheese to ripe, i.e. to form the desired texture, consistency and flavour of the cheese. The development of the texture and flavour is the result of complex physical and biochemical processes. These processes are influenced by a wide range of factors such as the chemical and structural composition in addition to the microbial flora in the raw milk, the starter culture added and the hygienic and manufacturing conditions used.

It has been shown that the activity of some strains of *Lactobacillus* species during the cheese ripening has an effect on the texture and flavour of the resulting cheese. Due to their capability to proliferate under cheese ripening conditions they form the major flora in cheese after a relatively short period of ripening (Fitzsimons et al., 2001). The lactobacilli found most frequently include mesophilic types such as *Lb. plantarum*, *Lb. casei*, *Lb. paracasei*, *Lb. brevis* and *Lb. curvatus* (Peterson and Marshall, 1990, Fitzsimons et al., 2001). Traditionally, various *Lactobacillus* species gain access to the cheese from the raw milk or the dairy plant environment, but due to their positive influence on the cheese flavour and texture selected cultures have been added to the cheese milk as adjunct cultures in order to influence the ripening process. Normally, lactobacilli are scarcely represented at the initial stages of the ripening process but, as mentioned above, when number of added starter culture organism has decreased, lactobacilli often become the dominant bacterial flora in the cheese, i.e. they grow from typical cell counts of $10-10^2$ to 10^7-10^8 CFU per gram of cheese during the first month of the ripening period (Ardö, 1993).

The presently known species of *Lactobacillus* are gram-positive, anaerobic or slightly aerobic, asporogenic and, depending upon the strain, *Lactobacillus* is in a coccus rod form, curved form, coryneform or filiform. Cells of known *Lactobacillus* species are non-motile, catalase-negative and do not reduce nitrates. Furthermore, they do not degrade gelatin and do not produce indole or hydrogen sulfide. They have normally weak caseinolytic and lipolytic activity, are strongly saccharolytic, acid tolerant and produce lactic acid.

As mentioned above, lactobacilli have an advantageous impact on the flavour intensity and the flavour characteristics of cheese. Their flavour-enhancing effect seems mainly to be due to the capability to boost the peptide breakdown in the cheese during ripening. Thus, lactobacilli are responsible for metabolising peptides and amino acids. Furthermore, the lactobacilli interacts positively with other starter cultures present in the cheese e.g. primary and/or secondary microbial starter cultures.

The length of the cheese ripening process normally varies from a few weeks to several months or years. Owing to the high costs of ripening facilities and stocks, the ripening process is expensive. Consequently, there is commercial interest in acceleration and control of ripening of cheese types that need long maturation. Control as used herein is to be understood as regulation of cheese ripening.

The prior art discloses many attempts to accelerate the cheese ripening. One such attempt is to increase the number of viable cells of the starter culture in the curd by applying high inoculation rates. However, this often resulted in an undesirably low pH and high moisture content, defects in texture and inconsistent cheese quality (Fox, 1998, El Soda, 1993). A further attempt for enhancing the cheese ripening (Fox, 1998) is to raise the ripening temperature, as microbiological, chemical and enzymatic reactions in cheese can be accelerated by raising the temperature. However, not all reactions are affected equally, which allows for unbalanced flavour to evolve. A number of methods to achieve accelerated cheese ripening are mainly based on enzyme addition or addition of attenuated bacterial cells. However, the risk of over-ripening still remains in the application of enzymes and, hence, it may be difficult to control the ripening process.

However, the effects of the known *Lactobacillus* species as adjunct cultures are not always predictable and controllable, and their use often involve side effects such as production of CO_2 gas from free amino acids. Thus, there is an industrial need for finding new species of *Lactobacillus* having improved characteristics and which are useful and reliable for accelerating cheese ripening and improving or enhancing the flavour of e.g. reduced fat or low fat cheese.

Now it has surprisingly been shown by the inventors of the present invention, that the cheese ripening process can be influenced by addition of *Lactobacillus danicus*, a novel *Lactobacillus* strain as adjunct cultures to the starter culture for production of cheese. Additionally, the inventors have demonstrated that an enhanced and/or improved flavour develops by addition of said novel *Lactobacillus* strain as detailed below.

SUMMARY OF THE INVENTION

The present invention provides bacterial strains belonging to *Lactobacillus danicus*, a novel *Lactobacillus* species. These new strains have been isolated from a product samples of Danish and Estonian cheese and from several traditional Danish and dairy starter cultures and have been analysed according to the principles of numerical taxonomy with respect to other *Lactobacillus* species in order to establish their novelty. In addition, the isolated bacteria have further been characterised by an analysis of their chemotaxonomic characteristics, which will be detailed below.

In a first aspect the present invention relates to an isolated lactic acid bacterium having the following characteristics:

a) forms long as well as short slender rods;
b) is non-motile;
c) grows at temperatures from 10°C to 37°C;
d) does not grow at 45°C;
e) grows in the presence of up to 10% NaCl;

f) capable of fermenting D-glucose, ribose, D-xylose, galactose, maltose, lactose and N-acetyl glucosamine;
g) not capable of fermenting amygdalin, D-arabinose, cellobiose, esculine, D-fructose, gluconate, mannitol, melezitose, melobiose, D-raffinose, rhamnose, saccharose, salicine, sorbitol, trehalose and D-manose.

In further aspect, the invention relates to an isolated lactic acid bacterium showing ≥93% 16S rRNA sequence similarity with the *L. danicus* strain 9M3 (DSM 14302) and to a pure culture of the lactic acid bacteria.

In a still further aspect, a culture composition is provided comprising a lactic acid bacterium according to the invention or a pure culture according to the invention.

The invention pertains in another aspect to the use of a lactic acid bacterium according to the invention or a pure culture according to the invention.

In a further aspect, the present invention relates to a method of preparing a food, feed, sweet or pharmaceutical product comprising adding an effective amount of the lactic acid bacterium according to the invention or a pure culture according to the invention or a culture composition according to the invention to a food, feed, sweet or pharmaceutical product starting material and keeping the thus inoculated starting material under conditions where the lactic acid bacterium is metabolically active.

The present invention also provides a fermented food product obtainable by the method according to the invention and a fermented feed product obtainable by the method according to the invention. Furthermore, the present invention provides a food product comprising a lactic acid bacterium according to the invention.

In a still further aspect, the present invention relates to a method for acceleration and controlling the ripening of a cheese, comprising the steps of (i) adding to cheese milk a lactic acid bacterium according to the invention or a pure culture according to the invention or a composition according to the invention; (ii) carrying out conventional cheese curd preparing steps; and (III) keeping the cheese curd resulting from step (II) under ripening conditions to obtain a ripened cheese.

DETAILED DISCLOSURE OF THE INVENTION

The inventors of the present invention have surprisingly isolated novel lactic acid bacteria having the above-mentioned properties. A phylogenetic analysis where the 16S rRNA sequence of the strain was compared with other lactic acid bacteria confirmed that the isolated strain belongs to *Lactobacillus danicus*, a new *Lactobacillus* species.

In accordance with one embodiment of the present invention, the isolated lactic acid bacterium is *L. danicus* strain 9M3 (DSM 14302). This bacterial strain and thus the new *Lactobacillus* species can be distinguished from other lactic acid bacteria by e.g. its carbohydrate utilization pattern and the below discussed 16S rRNA sequence. Furthermore, the strain 9M3 is able to grow at 10-37ºC but not at 45ºC, other strains of *L. danicus* are able to grow at 10-30ºC but not at 45ºC.

As further shown in the below Example, a nearly complete sequence of the 16S rRNA gene was determined and the sequence was used to search for 16S rRNA sequence similarity in known nucleotide databases. Comparison of 16S rRNA sequences, which are highly conserved among all organisms, can be used to assess the phylogenetic relationship between organisms. By the use of this technique, it was found by the inventors that the isolated lactic acid bacterium 9M3 was not closely related to any known bacteria, but the analysis revealed that the isolated strains belong to the genus *Lactobacillus*.

In yet a further aspect, the present invention relates to an isolated lactic acid bacterium showing at least 93% 16S rRNA sequence similarity with the strain 9M3 (DSM 14302). In preferred embodiments, the lactic acid bacteria of the present invention shows at least 93%, such as at least 94%, e.g. at least 95%, e.g. at least 96% e.g. at least 97%, including at least 98% or even at least 99% 16S rRNA sequence similarity with the strain 9M3 (DSM 14302).

The present invention also discloses an isolated, pure culture of the lactic acid bacterium having the above-mentioned characteristics. As used herein, the expression "pure culture" indicates that the culture contains a biomass of one single isolate of the lactic acid bacterium according to the invention, i.e. a clone originating in principle from one cell. Such a pure culture may be provided as a liquid cell suspension or as frozen, spray-dried or freeze-dried preparation. Preferably the pure culture is a concentrate of cells obtained by separation of cells e.g. by centrifugation or filtration using conventional techniques.

In yet a further aspect, the present invention relates to a culture composition comprising the above defined pure culture or lactic acid bacterium, and preferably a microbiologically acceptable carrier. One preferred embodiment is where the culture composition is an adjunct composition, i.e. a culture composition which can be used alone or in combination with attenuated cultures for influencing the flavour intensity and flavour character.

In one useful embodiment, the culture composition is a starter culture. It may be preferred that such a culture composition contains at least 10^3 colony forming units (CFUs) of the bacterium such as at least 10^4 CFUs per g, e.g. at least 10^5 CFUs per g, including at least 10^6 CFUs per g, such as at least 10^7 CFUs per g, e.g. at least 10^8 CFUs per g, including at least 10^9 CFUs per g, such as at least 10^{10} CFUs per g, such as at least 10^{11} CFUs per g, e.g. at least 10^{12} CFUs per g, including at least 10^{13} CFUs per g. Suitable carrier substances include nutrients such as an assimilable carbohydrate or a nitrogen source, which can be utilised readily by the lactic acid bacterium. Typically, such a composition is

provided in the form of a frozen or freeze-dried composition. In the latter case, the composition may contain cryoprotective compounds.

The composition may, in accordance with the invention, comprise two or more different species of organism or two or more strains of the same species. It is common in the production of food and feed products to apply mixed starter cultures, i.e. cultures comprising a multiplicity of strains. It is well known in the art that microbial interactions take place during fermentation. Such interactions can be positive interactions which have an impact on the overall performance of the fermentation e.g. by improving flavour and/or texture of the resulting fermentation product. In preferred embodiments, the culture composition further comprises a microorganism selected from the group consisting of a lactic acid bacterial species including *Lactococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp., *Weissella* spp., *Pediococcus* spp., *Oenococcus* spp. and *Streptococcus* spp., a *Bifidobacterium* species, a *Propionibacterium* species, a *Staphylococcus* species, a *Micrococcus* species, a *Bacillus* species, an *Enterobacteriaceae* species including *E. coli*, an *Actinomycetes* species, a *Corynebacterium* species, a *Brevibacterium* species, a *Pediococcus* species, a *Pseudomonas* species, a *Sphingomonas* species, a *Mycobacterium* species, a *Rhodococcus* species, a fungal species and a yeast species.

In a further aspect, the present invention relates to the use of the above defined lactic acid bacterium or a pure culture as a starter culture or an adjunct culture preferably in the production of a product selected from the group consisting of a pharmaceutical product, a food product, a feed product, a dairy flavour and a product for cheese flavouring. Another significant application of the starter culture or adjunct culture according to the invention is as so-called probiotics. In the present context, the term "probiotic" is to be understood as microbial cultures which, when ingested in the form of viable cells by humans or animals, confer an improved health condition, e.g. by suppressing harmful microorganisms in the gastrointestinal tract, by enhancing the immune system or by contributing to the digestion of nutrients. A typical example of such a probiotically active product is "sweet acidophilus milk".

In useful embodiments, the food product in which the above starter culture or adjunct culture can be used is selected from the group consisting of a milk-based product, a vegetable product, a meat product, a fruit juice, a wine and a bakery product. In preferred embodiments, the milk-based product is selected from the group consisting of a cheese, a yoghurt, a butter, an inoculated sweet milk and a liquid fermented milk product such as e.g. buttermilk or drinkable yoghurt.

A method is also achieved by the present invention for preparing a food, feed, sweet or pharmaceutical product based on the use of the above defined lactic acid bacterium, the above pure culture or the above culture composition. In its broadest aspect, such a method comprises the process that an effective amount of a lactic acid bacterium, a pure culture or a culture composition according to the invention are added to a food, feed,

sweet or pharmaceutical product starting material, and that this inoculated starting material is kept under conditions where the lactic acid bacterium is metabolically active.

In the present invention useful food product starting material includes any material which is conventionally subjected to a lactic acid bacterial fermentation step such as milk, vegetable materials, meat products, fruit juices, must, wines, doughs and batters. In further embodiments, the resulting fermented food product in the method of the invention is a dairy product such as cheese and buttermilk. In still further embodiments, the starting material is a starting material for an animal feed such as silage e.g. grass, cereal material, peas, alfalfa or sugar-beet leaf, where the bacterial culture is inoculated in the feed crop to be ensiled in order to obtain a preservation hereof, or in protein rich animal waste products such as slaughtering offal and fish offal, also with the aims of preserving this offal for animal feeding purposes.

As used herein, the term "milk" means any type of milk or milk component including e.g. cow's milk, human milk, buffalo milk, goat's milk, sheep's milk, dairy products made from such milk, or whey. The milk can be further processed to obtain a dairy product which is selected from the group consisting of cheese, yoghurt, butter, inoculated sweet milk and a liquid fermented milk product such as e.g. buttermilk or drinking yoghurt. Such further processing steps are carried out using conventional process steps.

Typically, the lactic acid bacterial strain is added to the starting material in a concentration of viable cells which is at least 10^3 CFU/g, such as at least 10^4 CFU/g, e.g. at least 10^5 CFU/g, including at least 10^6 CFU/g, such as at least 10^7 CFU/g including at least 10^7 CFU/g, e.g. at least 10^8 CFU/g, e.g. at least 10^{10} CFU/g, such as at least 10^{11} CFU/g, e.g. at least 10^{12} CFU/g, such as at least 10^{13} CFU/g of the starting material. In preferred embodiments, the bacterial strain is added to the starting material at a concentration in the range of 10^3 to 10^{13} CFU/g of the material, such as in the range of 10^5 to 10^9 CFU/g of the material, e.g. in the range of 10^6 to 10^8 CFU/g including in the range of 10^7 to 10^8 CFU/g of the starting material.

In accordance with the present invention, a fermented food product obtainable by the above method is also disclosed. Such fermented food products are preferably selected from the group consisting of a milk-based product, a vegetable product, a meat product, a fruit juice, a wine and a bakery product. Fermentation in this context is to be understood as a process by which the bacterial cell is able to obtain energy through the breakdown of glucose and other sugar molecules without using oxygen.

In a useful embodiment, the above milk-based product is a cheese. It is recognised in the dairy industry that the manufacturing of low fat cheese presents problems in that the flavour development is extremely slow and consequently such cheeses have a low consumer appeal. As mentioned above, a number of attempts to accelerate and enhance the flavour development of cheese having either low and high or normal fat content have been described.

It has now surprisingly been found by the inventors of the present invention that the addition of the isolated lactic acid bacterium according to the invention results in an improved and/or accelerated flavour development, i.e. cheese ripening, in different kinds of cheeses. In a preferred embodiment in the present invention, the cheese has a fat content of 20 to 40% and a moisture content of 30 to 55%. In a further embodiment, the cheese has a fat content of 3 to 20% and a moisture content of 30 to 55%.

It is also a part of the present invention to provide a fermented feed product, a sweet product and a pharmaceutical product obtainable by the above method. Sweet products also comprise confectionary in the present context. The fermented feed product, the sweet product and the pharmaceutical product obtained from the above method can be differentiated from other products by the presence of the lactic acid bacterium according to the invention.

In a further aspect, the present invention relates to a food product comprising the above defined lactic acid bacterium. In continuation of the above discussion regarding the taste and flavour enhancing effect of the present lactic acid bacterium, it may be preferred that the lactic acid bacterium is present in the food product in an amount which is sufficient to procure an improved taste and flavour of the food product. The food product is preferably a dairy product such as e.g. a cheese.

As also mentioned above, experiments have indicated that the lactic acid bacterium of the invention influences the cheese ripening process. Accordingly, in a useful embodiment, a sufficient amount of the lactic acid bacterium in the food product improves flavour development and/or accelerates the ripening of the cheese.

In a further aspect, the present invention relates to a method for controlling the ripening of a cheese, said method comprising a first step wherein a lactic acid bacterium, the pure culture or the culture composition as defined above is added to the cheese milk. Subsequently, conventional cheese curd preparing steps are carried out.

In a further step of the present method the cheese curd resulting from the above step is kept under ripening conditions to allow for development of a cheese. It is to be understood that the expression "ripening conditions" comprises conditions, which permit the cheese curd to ripe into a finished cheese. The temperatures for ripening are usually within the range of 2 to 30°C.

The invention will now be described in further details in the following non-limiting example with the drawing as shown in Fig. 1 showing the nearly complete sequence (1547 bp) of the 16S rRNA gene of strain 9M3 (SEQ ID NO 9).

EXAMPLE 1

Detailed description of the isolation and identification of strains belonging to the novel *Lactobacillus* species, *L. danicus*

1.1 Introduction

Mesophilic *Lactobacillus* species are normally not considered as part of starter cultures used in the cheese manufacturing, but they are often found in cheeses during ripening as a secondary flora. However, due to their advantageous influence on the cheese ripening process and the flavour of the resulting cheese *Lactobacillus* cultures are added in some cheese types such as Swiss cheeses and different Italian cheeses.

The object of the work leading to the present invention was to isolate lactic acid bacteria that had a beneficial influence on cheese ripening. A study of the microbial population in high quality cheeses during ripening resulted in the isolation of an unidentified bacterial strain. This strain was designated 9M3 and it was found that the strain belongs to a new *Lactobacillus* species.

1.2 Materials and methods

1.2.1 Bacterial strains and growth conditions
The bacterial strains investigated were isolated from Danish and Estonian cheeses and some traditional Danish dairy starter cultures. All organisms were grown on Rogosa agar and MRS agar or broth (Merck) at 30°C. Agar plates were incubated in anaerobic jars in a $CO_2 + H_2$ atmosphere (GasPac Systen, BBL). Liquid cultures were grown in test tubes in a normal atmosphere in freshly prepared growth medium.

1.2.2 Morphological, physiological and biochemical tests
Cell morphology was studied using phase-contrast microscopy. Gram staining was accomplished according to conventional methods such as e.g the instructions given in Preston, N.W. and Morell, A. "Reproducible results with Gram Stain", J. Path. Bact. 84, 241, (1962). The catalase activity was tested with a 3% (v/v) solution of hydrogen peroxide. The ability to ferment different carbohydrates was determined by the use of API 50 CH system (BioMérieux) according to the manufacturers instructions and the reactions were determined after an incubation period of 48 hours at a temperature of 30°C.

Gas production from the fermentation of glucose was determined in test tubes with MRS that, after inoculation, were overlaid with 0.8% agar and subsequently incubated at 30°C. The ability of the bacteria to tolerate and grow in the presence of NaCl was tested in test tubes with MRS broth supplemented with 6.5 and 10 % NaCl.

The formation of L-lactate and D-lactate from glucose fermentation was determined by use of a L-lactate and D-lactate dehydrogenase kit (Boehringer Mannheim).

1.2.3 16S rRNA sequencing

Genomic DNA was extracted from 1 ml freshly grown cultures. The cells were collected by centrifugation in an Eppendorf tube and resuspended in 0.4 ml TE buffer (50mM Tris, 1mmol l-1 EDTA, pH 8.0). An amount of 0.1 g glass beads (106 microns and finer, Sigma) was added and the cells were disrupted by shaking for 45 sec at 6.5 m/sec in FastPrep (Bio 101 Savant, NY, USA). The 16S rRNA gene of strain 9M3 was amplified by PCR using primer 16S-27F (5'-AGA GTT TGA TCM TGG CTC AG-3', SEQ ID NO:1) at the beginning of the 16S gene and the primer 23S-32R (5'-GCC ARG GCA TGG ACC-3', SEQ ID NO:2) at the beginning of 23S rRNA gene at position 32 to 47. PCR amplification was performed in 50 µl PCR buffer (Amersham Pharmacia, New Jersey, USA) containing 2 mM $MgCl_2$, 0.2 mM of each dNTP, 50 pmol of each primer, 2.5 u Taq polymerase and 1 µl of the extracted DNA. PCR condition was: 94°C for 5 min followed by 30 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 30 sec. The PCR products were purified using QIAquick PCRPurification kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The purified PCR products were sequenced directly using the primers 357R (5'-CTG CTG CCT CCC GTA GGA G-3', SEQ ID NO:3), 776F (5'-GAG CAA ACA GGA TTA GAT ACC-3', SEQ ID NO:4), 810R (5'-GCG TGG ACT ACC AGG GTA TCT-3', SEQ ID NO:5), 1050F (5'-GGC TGT CGT CAG GTG-3', SEQ ID NO:6), 1193R (5'-CGT CAT CCC CAC CTT CCT C-3', SEQ ID NO:7) and 1391F (5'-CTT GTA CAC ACC GCC CGT CA-3', SEQ ID NO:8) using a Dye Terminator Cycle Sequencing Kit (Beckman, USA) on an automatic capillary sequencer (CEQ 2000 DNA Analysing System, Beckman) with the primers. A nearly complete sequence (1547 bp) of the 16S rRNA gene was obtained from the strain 9M3 (Fig. 1, SEQ ID NO 9).

1.2.4 Phylogenetic analysis

The obtained DNA sequence of the 16S rRNA gene from 9M3 was used to search for similar sequences in Genbank with the program BLAST at NCBI (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov).

1.2.5 Selective media for isolation of these new bacteria

Most lactic acid bacteria used as starter cultures in the dairy industries are not able to utilize D-xylose as a carbon source. A selective culture medium containing D-xylose was composed in the present study to contain per l: Trypticase Peptone (BBL) 10g, Yeast extract (Oxoid) 5g, KH_2PO_4 6g, $MgSO_4*7H_2O$, 0.58g, $MnSO_4*4H_2O$ 0.15g $FeSO_4*7H_2O$ 0.03g and D-xylose 10g and it was autoclaved for 20 min at a temperature of 121°C.

1.2.6 Isolation of these new bacteria

Test tubes containing 10 ml of the above D-xylose medium were inoculated with 0.1 gram of a mixture of dairy-related strains of *Lactococcus* and *Leuconostoc* in an amount of 10^{11} cells per gram, together with strain 9M3 at a concentration of 10^3 cells per gram. The test tube was then incubated for 48 hours at 30°C. Subsequently 0.1 ml of this culture was used to inoculate a new test tub containing 10 ml of the D-xylose medium followed by incubation at 30°C for 48 hours. After incubation the culture was examined for the content of the strain 9M3 by plating on MRS plates and by microscopy

1.3 Results

1.3.1 Results of the morphological, physiological and biochemical tests
Strain 9M3 was analysed to be Gram-positive, non-motile, non-sporing, catalase negative an to form short to very long slender rods and to occur mainly as single cells. Cells longer than 50 µm have been observed. Colonies on MRS, Rogosa or BHI are rather small, 1 to 3 mm in diameter, round, rough edges, slightly transparent and white to creamy in colour.

The bacterium produced D-lactate and L-lactate from glucose and produced gas in late exponential growth phase. The strain 9M3 appears to be able to ferment D-glucose, ribose, D-xylose, galactose, maltose, lactose and N-acetyl glucosamine. Fermentation of galactose, maltose and N-acetyl glucosamine are variable or slow in some *L. danicus* strains. The strain 9M3 is furthermore able to grow at 10-37°C but not at 45°C, other isolated strains of *L. danicus* are able to grow at 10-30°C but not at 45°C. The strain 9M3 is able to grow in MRS broth containing 6.5%, 8%, 9% and 10% NaCl.

1.3.2 Results of the phylogenetic analysis
The bacterial strain 9M3 of the present invention is further characterised in that it has a DNA sequence of the 16S rRNA gene that relates it to the genus *Lactobacillus*. The results from the databases search showed that the known lactic acid bacterial species with the most similar 16S rRNA sequences were *Lactobacillus mucosae*, accession no. AF126738, *sp. nov.*(92.4%), *Lactobacillus pentosus*, accession No. D79211, (90.3%), *Lactobacillus plantarum*, accession No. D79210 (90.2%), *Lactobacillus casei*, accession no. D16553 (89.0%). Strain 9M3 also showed similarity to *Pediococcus inopinatus*, accession No. AJ271383 (88.1%). Thus, this result confirmed that the bacteria of the present invention belong to the genus *Lactobacillus*, but that it is clearly distinguishable from any of the known *Lactobacillus* species.

The strain 9M3 was deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Mascheroder Weg 1b, D-38124 Braunschweig, Germany on 17 May 2001 under the accession No. DSM 14302.

1.3.3 Conclusion of the experiments
It has been shown that it is possible to isolate a *Lactobacillus* species from a mixture of different lactic acid bacteria by using a medium containing D-xylose as the only carbon source in the medium.

The phylogenic analysis showed that the isolated *Lactobacillus* strain belongs to *L. danicus*, a novel *Lactobacillus* species not described before.

Applicant's or agent's file reference	International application No.
32631PC01	PCT/DK 02/00515

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page 4, line 8.

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution

DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

Address of depositary institution *(including postal code and country)*

Mascheroder Weg 1b
D-38124 Braunschweig

Date of deposit	Accession Number
2001-05-11	DSM 14302

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

As regards the respective Patent Office of the respective designated states, the applicant requests that a sample of the deposited microorganism only be made available to an expert nominated by the requester until the date on which the patent is granted or the date on wich the application has been refused or withdrawn or is deemed to be withdrawn.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g. "Accession Number of Deposit")*

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CLAIMS

1. An isolated lactic acid bacterium having the following characteristics:

a) forms long slender rods;
b) is non-motile;
c) grows at temperatures from 10°C to 37°C;
d) does not grow at 45°C;
e) grows in the presence of up to 10% NaCl;
f) capable of fermenting D-glucose, ribose, D-xylose, galactose, maltose, lactose and N-acetyl glucosamine;
g) not capable of fermenting amygdalin, D-arabinose, cellobiose, esculine, D-fructose, gluconate, mannitol, melezitose, melobiose, D-raffinose, rhamnose, saccharose, salicine, sorbitol, trehalose and D-manose.

2. 1. An isolated lactic acid bacterium according to claim 1 growing at temperatures from 10°C to 30°C.

3. An isolated lactic acid bacterium according to claim 1 or 2 which is *L. danicus* strain 9M3 (DSM 14302).

4. An isolated lactic acid bacterium showing at least 93% 16S rRNA sequence similarity with the strain 9M3 (DSM 14302).

5. A pure culture of the lactic acid bacterium according to claims 1 to 4.

6. A culture composition comprising a lactic acid bacterium according to any of claims 1 to 4 or a pure culture according to claim 5.

7. A culture composition according to claim 6 which further comprises a microorganism selected from the group consisting of a lactic acid bacterial species, a *Bifidobacterium* species, a *Propionibacterium* species, a *Staphylococcus* species, a *Micrococcus* species, a *Bacillus* species, an *Enterobacteriaceae* species including *E. coli*, an *Actinomycetes* species, a *Corynebacterium* species, a *Brevibacterium* species, a *Pediococcus* species, a *Pseudomonas* species, a *Sphingomonas* species, a *Mycobacterium* species, a *Rhodococcus* species, a fungal species and a yeast species.

8. A culture composition according to claim 7 wherein the lactic acid bacterial species is selected from the group consisting of *Lactococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp., *Oenococcus* spp., *Streptococcus* spp. and *weisella* spp.

9. Use of a lactic acid bacterium according to any of claims 1 to 4 or a pure culture according to claim 5 as a starter culture or an adjunct culture.

10. Use according to claim 9 wherein the starter culture or adjunct culture is used in the production of a product selected from the group consisting of a pharmaceutical product, a food product, a feed product, a sweet product, a dairy flavour product and a product for cheese flavouring.

11. Use according to claim 10 wherein the food product is selected from the group consisting of a milk-based product, a vegetable product, a meat product, a fruit juice, a wine and a bakery product.

12. Use according to claim 11 wherein the milk-based product is selected from the group consisting of a cheese, a yoghurt, a butter, an inoculated sweet milk and a liquid fermented milk product.

13. A method of preparing a food product, a feed product, a sweet product, a dairy flavour product, a product for cheese flavouring or a pharmaceutical product comprising adding an effective amount of the lactic acid bacterium according to any of claims 1 to 4 or a pure culture according to claim 5 or a culture composition according to any of claims 6 to 8 to a starting material for production of said products and keeping this inoculated starting material under conditions where the lactic acid bacterium is metabolically active.

14. A method according to claim 13 wherein the starting material is selected from the group consisting of milk, a vegetable material, a meat product, a must, a fruit juice, a wine, a dough and a batter.

15. A method according to claim 13 wherein the bacterial strain is added to the starting material at a concentration in the range of 10^3 to 10^{13} CFU/g or g of the material.

16. A fermented food product obtainable by the method of any of claims 13 to 15.

17. A fermented food product according to claim 16 which is selected from the group consisting of a milk-based product, a vegetable product, a meat product, a fruit juice, a wine and a bakery product.

18. A fermented food product according to claim 17 wherein the milk-based product is a cheese.

19. A fermented food product according to claim 18 wherein the cheese has a fat content of 20 to 40% and a moisture content of 30 to 55%.

20. A fermented food product according to claim 18 wherein the cheese has a fat content of 3 to 20% and a moisture content of 30 to 55%.

21. A fermented feed product obtainable by the method according to claim 13.

22. A food product comprising a food and the lactic acid bacterium according to claims 1 to 4.

23. A food product according to claim 22 wherein the lactic acid bacterium is present in an amount which is sufficient to produce an enhanced cheese flavour.

24. A food product according to claim 23 wherein the food product is a cheese.

25. A food product according to any of claims 23 to 24 wherein the amount of the lactic acid bacterium is sufficient to enhance the ripening of the cheese.

26. A method for controlling the ripening of a cheese, comprising the steps of

(i) adding to cheese milk a lactic acid bacterium according to any of claims 1 to 4 or a pure culture according to claim 5 or a culture composition according to any of claims 6 to 8;

(ii) carrying out conventional cheese curd preparing steps; and

(iii) keeping the cheese curd resulting from step (ii) under ripening conditions to obtain a cheese.

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GAGTTTGATC GCTGGCTCAG TGGATGGACG CTGGCGGTAT GCCTAATACA TGCAAGTCGA ACGCATTCGT
CGTTTTAGAT TGACGGTGCT TGCACCAGAT TGAAAAGACA TTTGAATGAG TGGCGGACGG GTGAGTAACA
CGTGGGTAAC CTGCCCAAAA GTGGGGGATA ACATTTGGAA ACAAATGCTA ATACCGCATA AAAGATTAGA
ACCGCATGGT TCTAATTTGA AAGATGGTTT CGGCTATCAC TTTTGGATGG ACCCGCGGCG TATTAGTTAG
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GTGAGACTGC CGGTGACAAA
CCGGAGGAAG GTGGGGACGA CGTCAAATCA TCATGCCCCT TATGACCTGG GCTACACACG TGCTACAATG
GACGGTACAA CGAGTTGCGA ACTCGCGAGA GTAAGCTAAT CTCTTAAAGC CGTTCTCAGT TCGGACTGTA
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GTTCCCGGGC CTTGTACACA CCGCCCGTCA CACCATGAGA GTTTGTAACA CCCAAAGTCG GTGGGGTAAC
CTTTTAGGAG CCAGCCGCCT AAGGTGGGAC AGATGATTAG GGTGAAGTCG TAACAAGGTA GCCGTAA

Fig 1